Plant Cell Rep (2005) 23:699–704
DOI 10.1007/s00299-004-0873-0

Snezana D. Ivic-Haymes · Ann C. Smigocki

Biolistic transformation of highly regenerative sugar beet (*Beta vulgaris* L.) leaves

Received: 1 April 2004 / Revised: 31 July 2004 / Accepted: 3 August 2004 / Published online: 5 November 2004

Abstract Leaves of greenhouse-grown sugar beet (*Beta vulgaris* L.) plants that were first screened for high regeneration potential were transformed via particle bombardment with the *uidA* gene fused to the osmotin or proteinase inhibitor II gene promoter. Stably transformed calli were recovered as early as 7 weeks after bombardment and GUS-positive shoots regenerated 3 months after bombardment. The efficiency of transformation ranged from 0.9% to 3.7%, and stable integration of the *uidA* gene into the genome was confirmed by Southern blot analysis. The main advantages of direct bombardment of leaves to regenerate transformed sugar beet include (1) a readily available source of highly regenerative target tissue, (2) minimal tissue culture manipulation before and after bombardment, and (3) the overall rapid regeneration of transgenic shoots.

Keywords *Beta vulgaris* · Biolistic · *uidA* · *npt*II · Particle bombardment

Abbreviations *GUS:* β-Glucuronidase · *IBA:* Indole-3-butyric acid · *Km:* Kanamycin · *uidA:* β-Glucuronidase gene

Introduction

Sugar beet (*Beta vulgaris* L.) is a significant industrial crop of the temperate zone, the worldwide production of which exceeded 240 million tons in 2000 (FAO 2000). Due to its high productivity, sugar beet is becoming increasingly interesting not only as a source of sugar but also as a possible "green bioreactor," i.e., for synthesis and accumulation of new metabolites in roots (Menzel et al. 2003; Sevenier et al. 1998). Improvements in various sugar beet traits such as sugar yield, disease resistance, and growth habit have been achieved through conventional breeding. However, many significant agronomic problems, including susceptibility to the fungal pathogen *Cercospora beticola* and the sugar beet root maggot, have not been solved by conventional breeding since the sources of genetic disease resistance in sugar beet are limited (Bosemark 1993; Setiawan et al. 2000; Smigocki et al. 2003).

Although the object of biotechnological studies for years, sugar beet is still considered recalcitrant to transformation, and a routine method for the regeneration of transgenic plants is lacking (Snyder et al. 1999; Zhang et al. 2001). Reported methods include *Agrobacterium*-mediated transformation of shoot-base tissues (Hisano et al. 2004; Lindsey and Gallois 1990), cotyledonary-node explants (Krens et al. 1996), shoot explants (Zhang et al. 2001), or embryogenic callus (D'Halluin et al. 1992; Zhang et al. 2001) and polyethylene glycol-mediated transformation of guard-cell protoplasts (Hall et al. 1996). Transformation frequencies for these methods ranged from 0.03% (Hall et al. 1996) to about 15% (Lindsey and Gallois 1990), depending on the explant type and breeding line used. However, most of these methods are labor-intensive and require a relatively long time for explant preparation and regeneration of transgenic plants. One example of this is the method of D'Halluin et al. (1992), which requires 2–3 months for the generation of seedling-derived callus for transformation, followed by sequential transfer of tissues to five different media throughout the procedure. In addition, most of the methods are not readily applicable in laboratories other than those in which they were developed (Krens et al. 1996; Snyder et al. 1999; Ivic-Haymes and Smigocki, unpublished results). Private companies are known to use *Agrobacterium*-mediated transformation of cotyledons (Fry et al. 1991) to generate transgenic plants. However, this method is not well-described and requires high input due to its

Communicated by K.K. Kamo

S. D. Ivic-Haymes · A. C. Smigocki (✉)
Molecular Plant Pathology Laboratory,
United States Department of Agriculture,
Agricultural Research Service,
Beltsville, MD, 20705, USA
e-mail: smigocka@ba.ars.usda.gov
Tel.: +1-301-5047118
Fax: +1-301-5045449

low efficiency (0.14%; Krens et al. 1996). The particle bombardment method (Snyder et al. 1999) that uses hypocotyl callus of a highly regenerative tissue culture clone REL-1 (Saunders 1998) was found not to be effective for transforming commercially important sugar beet breeding lines (Ivic and Smigocki 2001). In addition, this method entails several lengthy (2–3 months) and labor-intensive steps for plant material preparation, including a seed germination step that is often plagued by high levels (up to 90%) of microbial contamination (Ritchie et al. 1989). We have recently developed a method in which leaves of a breeding line, FC607, were used to produce highly regenerative callus suspension cultures for particle bombardment (Ivic and Smigocki 2003). Although transformed embryos and callus lines were obtained, no transgenic plants regenerated. This is possibly due to the long period (5 months at a minimum) of culture required for the regeneration of transformed tissues that has been associated with reduced organogenic potential (Cassells and Curry 2001).

In this report, FC607 and three other (FC709-2, C69, and Z731) sugar beet breeding lines were evaluated for regenerative callus production on excised leaf disks. Since sugar beet germplasm is highly heterogeneous (Bosemark 1993), many individual plants of each breeding line were tested. The FC607 leaf disks excised from greenhouse-grown plants with the highest regeneration potential were used as targets for particle bombardment with a *uidA* reporter gene. This reduced the time necessary for preparing the target tissues and cultivating the tissues after bombardment by 2 months, thereby allowing for a relatively rapid regeneration of transgenic plants. For recalcitrant crops such as sugar beet, whose efficiency of transformation is hard to improve, reducing the time required for the production of transgenic plants is a distinct advantage.

Materials and methods

Plant material

Sugar beet plants of breeding lines FC607 (Smith and Ruppel 1980), FC709-2 (Panella 1999), C69 (Lewellen 2000), and Z731 (R. Lewellen, Salinas, CA) were grown in the greenhouse at 25°C (day) and 22°C (night) under natural light for 6–30 months. For determination of the regeneration potential, leaf disks (9 mm) were excised from partially expanded (less than 40% of final size) leaves that had been surface sterilized in a 20% (v/v) commercial bleach [5.25% (w/v) sodium hypochlorite] and 0.01% (w/v) sodium dodecyl sulfate solution for 20 min and then washed five times with sterile water. Two leaf disks were placed per 100-mm petri plate containing 35 ml B1 medium (Ivic and Smigocki 2003). The leaf disks were incubated for 7 weeks at 31°C in the dark. A minimum of 15 leaf disks were excised from each individual plant tested. Between 12 and 33 individual plants (total 79 plants) of each breeding line were evaluated.

Plasmid DNA and particle bombardment

Transformation vectors carried the reporter gene *uidA* (*gusA*) fused to either the tobacco osmotin (Osm) or the potato proteinase inhibitor II (Pin2) gene promoter and the selectable marker gene neomycin phosphotransferase II (*npt*II) for kanamycin (Km) resistance fused to the nopaline synthase (NOS) promoter (Fig. 2a; Snyder et al. 1999). Plasmid DNA was purified using the Plasmid Maxi kit (Qiagen, Valencia, Calif., http://www1.qiagen.com).

Preliminary experiments were performed for optimizing the physical and biological DNA delivery parameters. Helium pressures of 900, 1,100, and 1,350 psi and target distances of 9.2 cm and 12.3 cm were tested. We also compared the bombardment of leaf disks to the bombardment of intact leaf halves followed by excision of the leaf disks. Either the adaxial or abaxial side was bombarded and placed facing up or down on the medium. The influence of the osmoticum in the medium was also evaluated. The following protocol was adopted for all subsequent experiments. Approximately 4 h before bombardment, leaf disks of line FC607 were excised as described above. Between 15 and 20 leaf disks were placed abaxial side up on 20 ml B1MM medium (B1 plus 44.6 g/l mannitol and 44.6 g/l sorbitol as osmoticum) that was poured on top of a filter paper placed in a 100-mm petri plate (Sanford et al. 1993). Leaf disks were bombarded from a distance of 12.3 cm with gold particles (1.6 μm in size; Bio-Rad, Hercules, Calif., http://www.bio-rad.com) coated with plasmid DNA using 1,100-psi or 1,350-psi rupture disks (Biolistic Particle Delivery System PDS-1000/He, Bio-Rad) and cultured as above. To gradually reduce the osmoticum concentration (Sanford et al. 1993), filters with the leaf disks on the B1MM medium were transferred at 16 h and 24 h after bombardment to 10 ml and 20 ml of B1 medium, respectively.

Selection of transformed tissues

Two days after bombardment, leaf disks (two per plate) were transferred to B1 medium (35 ml) supplemented with 0, 10, 20, 25, 30 or 100 mg/l Km. The plates were incubated at 31°C in the dark for 7–16 weeks. Regenerated calli and shoots were maintained on B1 medium at 25°C under a 16/8-h (light/dark) photoperiod with light provided by cool-white fluorescent lighting (30 μmol/m^2 per second). For root induction, shoots (10 mm in height) were placed on MSB medium (B1 medium without benzylaminopurine) supplemented with 50 mg/l or 100 mg/l indole-3-butyric acid (IBA) for a 24-h period (Ivic et al. 2001). The shoots were then transferred to MSB medium without plant growth regulators and cultured at 25°C under continuous light. Rooted plantlets were transferred to Jiffy Mix potting soil (Jiffy Products, http://www.jiffyproducts.com) and acclimated for 2 weeks in a growth chamber at 25°C (day) and 22°C (night) under a 16/8-h (light/dark) photoperiod with light provided by cool-white fluorescent lighting (200 μmol/m^2 per second). The plants were transplanted into Fafard no. 52 soil mix (Conrad Fafard, Agawam, Mass., http://www.fafard.com) and grown in the greenhouse at 25°C (day) and 22°C (night) under natural light.

Histochemical analysis of GUS expression

GUS activity was assayed by incubating the tissues in an X-Gluc (5-bromo-4 chloro-3-indolyl-β-D-glucuronic acid) solution at 37°C overnight (Jefferson et al. 1987). After destaining in 70% ethyl alcohol, tissues were examined for *uidA* expression using a dissecting microscope.

Molecular analyses

Plant DNA was purified according to Haymes (1996). Ready To Go PCR beads (Amersham Biosciences, Piscataway, N.J., http://www4.amershambiosciences.com) were used for PCR analysis using gene-specific primers for *uidA* (forward primer, 5′-GGT CAG TCC CTT ATG TTA CG-3′; reverse primer, 5′- GTG TAG AGC ATT ACG CTG CG-3′) and *npt*II (forward primer, 5′-GAG GCT ATT CGG CTA TGA CTG-3′; reverse primer, 5′-ATC GGG AGC

GGC GAT ACC GTA-3′) that amplify a 544-bp and a 700-bp fragment, respectively. The PCR cycling profile consisted of an initial denaturation at 94°C for 4 min, 30 cycles of 94°, 55°, and 72°C for 1 min each, followed by a 7-min extension at 72°C carried out in a thermocycler (Eppendorf Scientific, Westbury, N.Y., http://www.eppendorf.com). Reaction products were subjected to electrophoresis on a 1% agarose gel.

For Southern blot hybridization analysis, 10 μg of plant DNA was digested with restriction enzymes, separated on a 0.8% (w/v) agarose gel, and blotted onto a positively charged nylon membrane using 20× SSC (0.3 *M* sodium citrate, 3 *M* sodium chloride). Blots were hybridized with a 0.54-kb *uidA*-specific probe labeled with digoxygenin-dUTP using a PCR DIG Probe Synthesis kit (Roche Applied Science, Indianapolis, Ind., http://www.roche-applied-science.com). Hybridization signal generation and detection were performed using chemiluminescent substrate as described by Roche. Blots were exposed to an X-omat AR film (Kodak, Eastman, N.Y., http://www.kodak.com) for 25 min.

Results and discussion

Sugar beet breeding lines differ in their ability to form regenerative callus on isolated leaf disks (Saunders and Doley 1986). Using the leaf-disk method, we evaluated the regeneration potential of four breeding lines selected on the basis of their relatively high sugar content and disease resistance characteristics (Lewellen 2000; Panella 1999; Smith and Ruppel 1980).

Leaf disks excised from all four breeding lines expanded during the first week of culture. Hard, white, nonregenerative calli appeared on the wounded edges of some leaf disks 3 weeks after isolation. At 5 weeks of culture, friable, yellow calli began to appear, some of which regenerated somatic embryos and adventitious shoots. The occurrence and speed of formation of regenerative callus varied both among lines and different plants of each line. The highest frequency of plants with regenerative callus (number of plants with regenerative callus per number of plants tested) at 7 weeks was obtained with line FC607 (61%; 20 of 33 tested), while none of the plants from line FC709-2 produced regenerative callus (Table 1). In addition, breeding line FC607 had the highest frequency (55.6%, Table 1) of regenerative callus production on leaf disks excised from the 20 plants that were determined to have a positive regeneration response. The range of regeneration frequencies for individual sugar beet plants within each breeding line was wide (Table 1). Individual plants with a relatively high regeneration frequency (69%, 75%, and 100%) were identified for the Z731, C69 and FC607 breeding lines, respectively. Leaves of highly regenerative FC607 plants were used for transformation experiments.

Leaf tissues were bombarded with gold particles coated with the *uidA* gene fused to the wound-inducible Osm or Pin2 gene promoters, which have been shown to induce relatively high levels of gene expression in biolistically treated sugar beet cell suspensions (Ingersoll et al. 1996; Ivic and Smigocki 2003). Two days after bombardment DNA delivery frequencies ranged from 2 GUS-positive units to 170 GUS-positive units (blue spots) per bombarded plate of 15–20 explants. High variability in transient gene expression is common for particle bombardment and most likely due to the variations in the degree of coating of the gold particles with the DNA (Sawant et al. 2000). The observed transient expression frequencies were lower than those previously reported for particle bombardment of sugar beet suspension cultures using the same genetic constructs (Ivic and Smigocki 2003), and this reduced transformation frequency may be attributed to the difference in the degree of penetration of the gold particles into the leaf tissue versus the suspension cells. The thick cuticular layer on leaf surfaces may reduce the number of particles that enter the cells or the depth of penetration. No significant difference was observed in the number of GUS-positive units per plate between plates bombarded at 1,100 psi or 1,350 psi (data not shown). A relatively small number of transiently expressed cells, however, may not be a disadvantage, since in some reports no correlation has been found between the frequencies of transient expression and stable transformation (Altpeter et al. 1996; Christou 1995).

Antibiotic selection, which allows for specific enrichment for transformed cells, is generally considered to be an integral part of a transformation protocol. A widely used selection scheme incorporates an *npt*II selectable marker gene into the targeted cells thereby making them resistant to Km and other related antibiotics. In some plants, however, selection of transformed cells with Km severely represses the subsequent regeneration of transgenic plants (Everett et al. 1987; Mullins 1990). The use of Km for selecting transformed sugar beet tissues has also proven to be difficult (Ivic and Smigocki 2003).

Table 1 Influence of genotype on regenerative callus production in leaf disks cultured for 7 weeks on B1 medium (*SE* standard error)

Breeding line	Percentage of plants producing regenerative callus	Percentage of explants with regenerative callus	
		Mean ± SE[a]	Range[b]
FC607	61 (20/33)[c]	55.6±5.4[d]	18.7–100.0
FC709-2	0 (0/14)	–	–
C69	35 (7/20)	14.1±10.2	3.0–75.3
Z731	50 (6/12)	26.4±9.2	7.7–69.2

[a] Mean percentage of explants was calculated by averaging the values obtained for individual plants that produced regenerative callus
[b] The lowest and the highest values for individual plants
[c] Number of plants producing regenerative callus/number of plants tested
[d] Significantly different at P=0.05 according to Fisher's LSD test. Percentage data were transformed to arcsin before analysis

Table 2 Transformation efficiency of sugar beet leaf disks bombarded with the Osm-*uidA* and Pin2-*uidA* gene constructs using 1,100 psi (T1 and T3) or 1,350 psi (T2 and T4)

Construct	Experiment	Km concentration (mg/l)	Number of explants	Explants with callus[a] (%)	GUS-positive		Transformation efficiency[b] (%)
					Callus	Shoots[c]	
Osm-*uidA*	T1	0	34	100[d]	1	0	–
	T2	0	27	48[d]	1	3	3.7[e]
		100	6	0	0	0	–
Pin2-*uidA*	T3	0	115	66[d]	1	1	0.9
	T4	0	32	100[d]	1	1	3.1
		10	3	67[f]	0	0	–
		20	6	17[f]	0	0	–
		25	8	50[f]	2	0	–
		30	8	38[f]	0	0	–

[a] Seven weeks after transformation
[b] Number of GUS-positive shoots/number of bombarded leaf disks
[c] Regenerated from GUS-positive calli
[d] Large, friable callus with shoots
[e] Corrected for a single independent transformation event (see Fig. 2c)
[f] Small, friable callus, no shoots

Therefore, most of the bombarded sugar beet explants were transferred to B1 medium without Km, while a smaller number of explants were transferred to B1 medium with 10–100 mg/l Km (Table 2). Since preliminary experiments showed that the periodic transfer of control (nonbombarded) leaf disks to fresh medium had a negative impact on regeneration (data not shown), explants were maintained on the same media without transfer for 16 weeks.

As with control explants, bombarded leaf disks expanded during the first week of culture, and friable callus began to appear on the wounded edges 5 weeks after bombardment when cultured on medium without Km. By week 7, 48–100% of these explants formed large (10–15 mm), friable, yellow calli (Table 2), 85% of which regenerated shoots (Fig. 1a) and embryos. The regeneration of calli was delayed and the calli grew slower when leaf disks were cultured on B1 medium supplemented with 10–30 mg/l Km; after 7 weeks, only a few explants formed one to two small (3 mm in diameter) friable calli per disk, but no shoots formed. After 16 weeks in culture, 24% of the explants regenerated calli with shoots, presumably after the Km began to break down in the media. A delay in the regeneration of transformed calli on Km-containing media was also observed when sugar beet suspension cultures were bombarded with the same gene constructs (Ivic and Smigocki 2003). No calli or shoots regenerated on explants cultured for 16 weeks on 100 mg/l Km. The overall contamination rate for bombarded leaf disks was 0.7%.

Portions of each recovered callus and all regenerated shoots were subjected to histochemical GUS test to check for expression of the *uidA* gene. GUS activity was detected in six calli regenerated from six different explants (Table 2), while none of the shoots expressed the *uidA* gene. Four of the GUS^+ calli regenerated 7–11 weeks following bombardment on medium not supplemented with Km, while the other two were obtained 14 weeks and 16 weeks after bombardment on Km-containing media (25 mg/l). All GUS-positive calli exhibited chimeric expression of the *uidA* gene—i.e., they were sectored for the presence of blue color. Moreover, the intensity of the blue color varied between different callus lines. It is possible that chimeric calli arose from both transformed and normal cells or that the *uidA* gene became silenced in certain

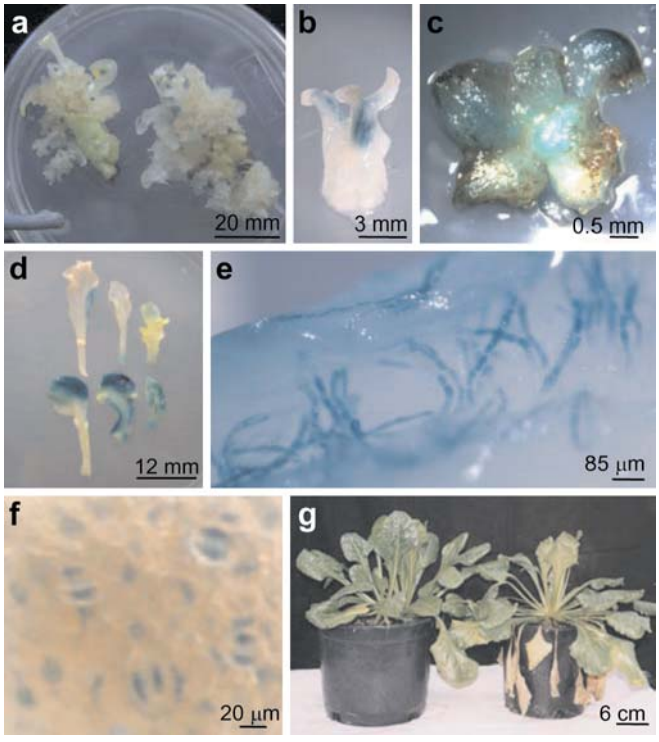

Fig. 1 **a** Regeneration of calli and shoots from FC607 leaf disks on B1 medium 7 weeks after bombardment with the Osm-*uidA* gene construct. GUS activity in Osm-*uidA* shoot (**b**), Pin2-*uidA* shoot (**c**), leaves (**d**) and trichomes (**e**) of tissue culture-propagated Osm-*uidA* plantlets, **f** epidermal and stomatal cells of greenhouse-grown Osm-*uidA* plants. **g** Osm-*uidA* (*left*) and nontransformed, control plant (*right*) 10 months after root induction

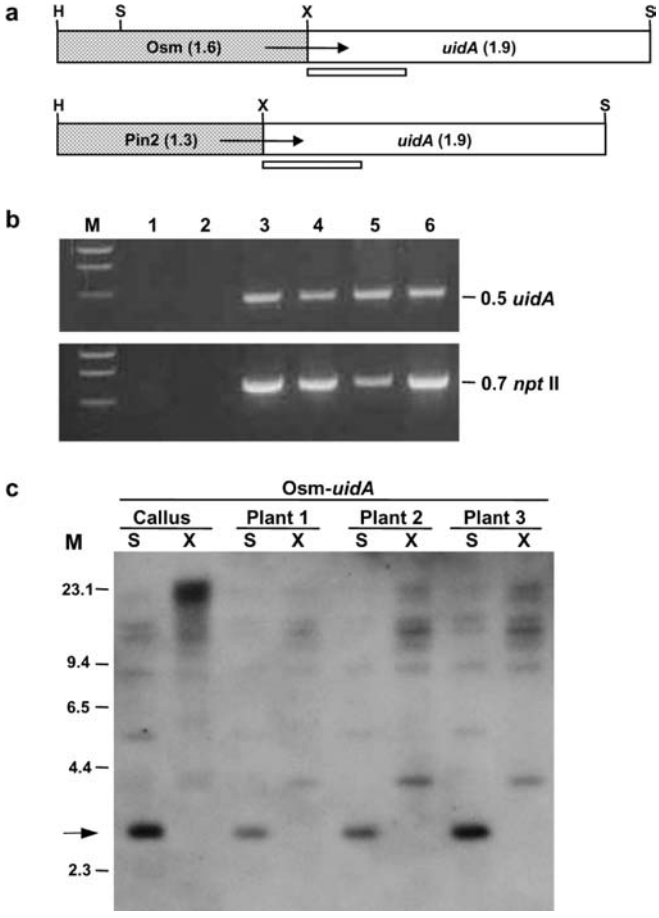

Fig. 2 **a** *uidA* gene constructs used to transform sugar beet leaf disks. The *uidA* gene was fused to either the Osm or Pin2 promoter. The probe used for Southern blot hybridization in **c** is indicated with a *bar*. *H HindIII*, *S SacI*, *X XbaI*. **b** PCR amplification of the *uidA* (0.5 kb) and *npt*II (0.7 kb) gene fragments in Osm-*uidA* transformed callus and shoots. *Lanes*: *1* Nontransformed leaves, *2* nontransformed callus, *3* callus from experiment T2, *4–6* three GUS-positive shoots regenerated from the callus in *lane 3*, *M* ØX174/*Hae*III molecular-weight marker. **c** Southern blot hybridization with a 0.54-kb digoxygenin-labeled *uidA* gene fragment as probe. Genomic DNA was digested with *Sac*I (*S*) that cuts at both ends of the promoter-gene construct to show the predicted fragment size of Osm-*uidA* (2.8 kb) (*arrow*) and with *Xba*I (*X*) with a single site within the promoter-gene construct to show the number of genomic integration sites of the transgene. *M* λ/*Hin*dIII molecular-weight marker (in kilobases)

parts of the transformed calli. Gene silencing and variation in the expression levels of the introduced gene are common in particle bombardment and may be due to a number of factors, including copy number of the introduced gene and positional effects (Reddy et al. 2003).

The GUS-positive calli were transferred to fresh shoot-inducing B1 medium and maintained as separate lines. Within 4–8 weeks after transfer, five of the six callus lines regenerated shoots, which were then tested for *uidA* gene expression by histochemical GUS assay. The GUS-positive line derived from experiment T2 regenerated 13 shoots, three of which were GUS-positive (Table 2, Fig. 1b). Callus lines from experiments T3 and T4 each regenerated one GUS-positive shoot (out of a total of one and ten shoots, respectively) (Table 2, Fig. 1c). The efficiency of transformation, calculated as the number of GUS-positive shoots per number of bombarded leaf disks, ranged from 0.9% to 3.7% (Table 2).

Only Osm-*uidA* transgenic shoots from experiment T2 were micropropagated and transferred to rooting media since the Pin2-*uidA* shoots were sacrificed for the GUS analysis. Transformants rooted at a frequency of 44% and 100% after a 24-h exposure to 50 mg/l or 100 mg/l IBA, respectively, in comparison to a rooting frequency of 95% for the untransformed, control shoots. Rooted transgenic plantlets had lower survival rates than the control plants when they were transferred to soil, possibly due to the less prolific root development and overall shorter roots. The overall growth characteristics of the transgenic plants that survived, however, were similar to those of the non-transformed control plants (Fig. 1f).

GUS activity in tissue culture-propagated Osm-*uidA* shoots from experiment T2 was detected in leaves, petioles, trichomes (Fig. 1b,d,e) and roots (not shown). A significant difference was observed in the *uidA* expression pattern in different leaves of the same plantlet (Fig. 1d): some leaves were completely blue, while some did not show the presence of a blue color. In most cases, however, GUS activity was limited to the leaf tips and edges. This coloration pattern did not correlate with age or position of the leaves on the plantlet. A similar pattern was observed in hydroponically grown tobacco plants transformed with the Osm-*uidA* gene construct, where the strongest expression was observed in leaf tips, trichomes, and epidermal cells (Kononowicz et al. 1992).

Osm-*uidA* gene expression in greenhouse-grown plants appeared to be limited to the trichomes and epidermal and stomatal cells within small areas of the leaf (Fig. 1f). Unlike in the tissue culture-propagated shoots, GUS activity was not detected in the petioles and roots. No induction of *uidA* gene expression was detected when the leaves were wounded 12 h prior to GUS analysis, even though the Osm promoter has been shown to be wound-inducible in many plants, among them sugar beet (Snyder et al. 1999).

PCR analysis of the GUS-positive shoots from experiment T2 revealed the expected DNA fragment for both the *uidA* (0.5 kb) and *npt*II (0.7 kb) genes (Fig. 2b), and integration of the *uidA* gene into the genome was confirmed by Southern blot analysis (Fig. 2c). The expected 2.8-kb Osm-*uidA* promoter-gene fragment and other size fragments were detected, indicating possible rearrangements of the *uidA* gene (Fig. 2c; *Sac*I digest). Multiple integrations of the *uidA* gene were detected in the *Xba*I-digested DNA. The three transformed plants from experiment T2 showed an identical hybridization pattern, indicating that they were clones arising from a single transformation event.

In conclusion, a method was developed for generating genetically transformed sugar beet plants from a commercial sugar beet breeding line. Leaves of greenhouse-grown FC607 plants that were determined to have a high

regeneration potential proved to be a readily available source of target tissue for the particle bombardment method. Avoidance of the use of embryogenic callus and suspension cultures as targets for particle bombardment as well as the minimal (less than 1%) contamination rate of the cultured leaf disks provided a simple and time-saving procedure by which transformed plants were obtained as early as 3 months following bombardment. For recalcitrant crops such as sugar beet, whose efficiency of transformation is hard to improve, reducing the time required for the production of transgenic plants is an advantage (Altpeter et al. 1996). In addition, rapid production of transformed plants can prevent phenotypic abnormalities and reduced fertility caused by long periods in tissue culture (Cassells and Curry 2001; O'Kennedy et al. 2001).

References

Altpeter F, Vasil V, Srivastava V, Stöger E, Vasil I (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep 16:12–17

Bosemark NO (1993) Genetics and breeding. In: Cooke DA, Scott RK (eds) The sugar beet crop: science into practice. Chapmann & Hall, London, pp 37–66

Cassells AC, Curry RF (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tissue Organ Cult 64:145–157

Christou P (1995) Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. Euphytica 85:13–27

D'Halluin K, Bossut M, Bonne E, Mazur B, Leemans J, Botterman J (1992) Transformation of sugarbeet (*Beta vulgaris* L.) and evaluation of herbicide resistance in transgenic plants. Biotechnology 10:309–314

Everett NP, Robinson KEP, Mascarenhas D (1987) Genetic engineering of sunflower (*Helianthus annuus* L.). Biotechnology 5:1201–1204

FAO (2000) Production Yearbook. FAO Statistics Series, No. 163, Rome

Fry JE, Barnason AR, Hinchee M (1991) Genotype-independent transformation of sugarbeet using *Agrobacterium tumefaciens*. In: Hallick RB (ed) Molecular biology of plant growth and development. Third Int Congr Int Soc Plant Mol Biol. Tuckon, USA Int Soc Plant Mol Biol, Athens, p 384

Hall RD, Riksen-Grunisma T, Weyens GJ, Rosquin IJ, Denys PN, Evans IJ, Lathouwers JE, Lefebvre MP, Dunwell JM, van Tunen A, Krens FA (1996) A high efficiency technique for the generation of transgenic sugarbeet from stomatal guard cells. Nat Biotechnol 14:1133–1138

Haymes KM (1996) A DNA mini-prep method suitable for a plant breeding program. Plant Mol Biol Rep 14:280–284

Hisano H, Kimoto Y, Hayakawa H, Takeichi J, Domae T, Hashimoto R, Abe J, Asano S, Kanazawa A, Shimamoto Y (2004) High frequency *Agrobacterium*-mediated transformation and plant regeneration via direct shoot formation from leaf explants in *Beta vulgaris* and *Beta maritima*. Plant Cell Rep 22:910–918

Ingersoll JC, Heutte TM, Owens LD (1996) Effect of promoter-leader sequences on transient expression of reporter gene chimeras biolistically transferred into sugarbeet (*Beta vulgaris* L.) suspension cells. Plant Cell Rep 15:836–840

Ivic SD, Smigocki AC (2001) Evaluation of the biolistic transformation method for commercially important sugar beet breeding lines. In: Proc 31st Meet Am Soc Sugar Beet Technol, Denver, p 207

Ivic SD, Smigocki AC (2003) Transformation of sugar beet cell suspension cultures. In Vitro Cell Dev Biol Plant 39:573–577

Ivic SD, Sicher RC, Smigocki AC (2001) Growth habit and sugar accumulation in sugar beet (*Beta vulgaris* L.) transformed with a cytokinin biosynthesis gene. Plant Cell Rep 20:770–773

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Kononowicz AK, Nelson DE, Singh NK, Hasegawa PM, Bressan RA (1992) Regulation of the osmotin gene promoter. Plant Cell 4:513–524

Krens FA, Trifonova A, Keizer LCP, Hall RD (1996) The effect of exogenously applied phytohormones on gene transfer efficiency in sugarbeet (*Beta vulgaris* L.). Plant Sci 116:97–106

Lewellen RT (2000) Registration of multiple disease resistant C69 sugarbeet germplasm. Crop Sci 40:1511

Lindsey K, Gallois P (1990) Transformation of sugarbeet (*Beta vulgaris* L.) by *Agrobacterium tumefaciens*. J Exp Bot 41:529–536

Menzel G, Harloff HJ, Jung C (2003) Expression of bacterial poly(3-hydroxybutyrate) synthesis genes in hairy roots of sugar beet (*Beta vulgaris* L.). Appl Microbiol Biotechnol 60:571–576

Mullins MG (1990) *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. Biotechnology 8:1041–1045

O'Kennedy MM, Burger JT, Berger DK (2001) Transformation of elite white maize using the particle inflow gun and detailed analysis of a low-copy integration event. Plant Cell Rep 20:721–730

Panella L (1999) Registration of FC709-2 and FC727 sugarbeet germplasm resistant to Rhizoctonia root rot and Cercospora leaf spot. Crop Sci 39:298–299

Reddy MSS, Dinkins RD, Collins GB (2003) Gene silencing in transgenic soybean plants transformed via particle bombardment. Plant Cell Rep 21:676–683

Ritchie GA, Short KC, Davey MR (1989) In vitro shoot regeneration from callus, leaf axils and petioles of sugar beet (*Beta vulgaris* L.). J Exp Bot 40:277–283

Sanford JC, Smith FD, Russell JA (1993) Optimizing biolistic process for different biological applications. Methods Enzymol 217:483–509

Saunders JW (1998) Registration of REL-1 and REL-2 sugarbeet germplasms for tissue culture genetic manipulations. Crop Sci 38:901–902

Saunders JW, Doley WP (1986) One step shoot regeneration from callus of whole plant leaf explants of sugarbeet lines and a somaclonal variant for in vitro behavior. J Plant Physiol 124:473–479

Sawant SV, Singh PK, Tuli R (2000) Pretreatment of microprojectiles to improve the delivery of DNA in plant transformation. Biotechniques 29:246–248

Setiawan A, Koch G, Barnes SR, Jung C (2000) Mapping quantitative trait loci (QTLs) for resistance to *Cercospora* leaf spot disease (*Cercospora beticola* Sacc.) in sugar beet (*Beta vulgaris* L.). Theor Appl Genet 100:1176–1182

Sevenier R, Hall RD, van der Meer IM, Hakkert HJC, van Tunen AJ, Koops AJ (1998) High level fructan accumulation in a transgenic sugar beet. Nat Biotechnol 16:843–846

Smigocki AC, Campbell LG, Wozniak CA (2003) Leaf extracts from cytokinin-overproducing transgenic plants kill sugarbeet root maggot larvae. J Sugar Beet Res 40:197–207

Smith GA, Ruppel EG (1980) Registration of FC607 and FC607 CMS sugarbeet germplasm. Crop Sci 20:419

Snyder GW, Ingersoll JC, Smigocki AC, Owens LD (1999) Introduction of pathogen defense genes and a cytokinin biosynthetic gene into sugarbeet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment. Plant Cell Rep 18:829–834

Zhang CL, Chen DF, McCormac AC, Scott NW, Elliott MC, Slater A (2001) Use of the GFP reporter gene as a vital marker for *Agrobacterium*-mediated transformation of sugar beet (*Beta vulgaris* L.). Mol Biotechnol 17:109–117